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Mutations in the Cytoplasmic Domain of the Fusion Glycoprotein of Newcastle Disease Virus Depress Syncytia Formation

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The role of the cytoplasmic domain of the Newcastle disease virus fusion protein in syncytia formation was explored by characterizing the intracellular processing and activities of proteins with deletions and point mutations in this region. Deletion of the entire domain (amino acids 523 to 553) resulted in a protein which was minimally proteolytically cleaved and had no syncytia forming activity. Deletion of the carboxy terminal half of the domain (amino acids 540 to 553) resulted in a protein that was normally processed but had no syncytia forming activity. Deletion of amino acids 547 to 553 resulted in a protein with approximately 30% wild-type levels of activity while deletion of amino acids 550 to 553 yielded a protein with wild-type activity. The results suggested that amino acids 540 to 550 are important for syncytia formation and this conclusion was supported by two internal deletions as well as point mutations in this region. Mutation of two cysteine residues in and adjacent to the transmembrane domain, which are potential sites for fatty acid acylation, had no effect on syncytia formation either singly or in combination.

INTRODUCTION

Enveloped viruses gain entry into cells by membrane fusion, and all enveloped viruses encode a surface glycoprotein which mediates this fusion. These proteins share some common structural features important to the activity of the protein. Among these features are a fusion peptide rich in short chain amino acids and one or several heptad repeat sequences (Chambers *et al.*, 1990; White, 1990, 1992). Many fusion glycoproteins are also synthesized as a precursor which must be proteolytically cleaved to activate the fusion activity (White, 1990).

Fusion proteins from viruses which infect cells in a pH independent fashion also have the property of inducing cell to cell fusion or syncytia formation (White, 1990, 1992). Recent studies of several different viral fusion proteins have shown that the cytoplasmic domain may influence the formation of syncytia. Mason–Pfizer monkey virus and MuLV TM (transmembrane) proteins are transported to the cell surface with long cytoplasmic domains and little syncytia forming activity. After budding, the cytoplasmic domain of the protein is cleaved by a virion protease to produce the mature TM protein which is active in fusion (Brody *et al.*, 1993; Rein *et al.*, 1994). The TM protein of SIV isolated from infected animals has low syncytia forming activity and a long cytoplasmic tail. As the virus is propagated in tissue culture, mutants are selected which contain a polypeptide chain termination codon which results in a specific truncation of the cytoplasmic domain of the TM protein (Chakrabarti *et al.*, 1989; Hirsch *et al.*, 1989) and much increased syncytium forming activity (Ritter *et al.*, 1993). Other cytoplasmic domain truncations of the SIV TM glycoprotein, however, reduce syncytia formation (Spies and Compans, 1994). Some truncations of the HIV TM protein can increase syncytia formation (Dubay *et al.*, 1992). Truncations of the glycoprotein H of herpes simplex I virus reduce syncytia formation (Wilson *et al.*, 1994). These combined results strongly suggest that the cytoplasmic domain of a fusion protein can modulate syncytia formation either positively or negatively, albeit by unknown mechanisms.

These observations raise the question of the role of the cyoplasmic domain in the activities of other fusion proteins. The paramyxovirus fusion proteins, classic fusion proteins, are synthesized as a precursor F_0 which is proteolytically cleaved into F_1 and F_2 activating fusion activity. The proteins have a fusion peptide and two heptad repeat regions, one located adjacent to the fusion peptide and the other adjacent to the transmembrane domain (Buckland *et al.*, 1992; Chambers *et al.*, 1990; Lamb, 1993). While mutational analyses of these domains have demonstrated roles in fusion (Buckland *et al.*, 1992; Horvath and Lamb, 1992; Sergel-Germano *et al.*, 1994), there has been no systematic mutational analysis of the cytoplasmic domain, although characterization of several truncated measles virus F proteins derived from SSPE brains have indicated that the cytoplasmic domain may influence syncytia formation in measles virus systems (Cattaneo and Rose, 1993). We describe here the properties of deletions and point mutations in the cytoplasmic domain of the fusion protein of Newcas-

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tle disease virus (NDV). Deletions do not increase syncytia formation but rather decrease the activity of the protein. Results suggest that the more carboxy terminal region of the cytoplasmic domain is most important for syncytia formation. Furthermore, results suggest that the cytoplasmic domain may influence the conformation of the protein.

MATERIALS AND METHODS

Cells, vectors, and viruses

Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids, vitamins, penicillin/streptomycin, and 10% fetal calf serum.

NDV HN and F genes, characterized previously (Sergel *et al.,* 1993), were expressed in Cos cells using pSVL obtained from Pharmacia. Viral genes were inserted into *Sac*I and *Xba*I cut plasmid DNA.

NDV (strain AV) was grown in eggs and purified by standard protocols.

Site-specific mutagenesis

The F gene was ligated into an *Xba*I and *Sac*I cut M13-mp10 for mutagenesis. Single-stranded template was prepared and mutagenesis reactions were done using the Sculptor mutagenesis kit from Amersham Corp. The appropriate oligomers of 17–38 nucleotides in length were used for each mutation. Several plaques were picked and the single-stranded phage DNAs were sequenced to identify mutants. The entire gene of the mutant DNA was sequenced to verify that the rest of the gene remained unchanged by the mutagenesis reaction. The replicative form DNA of the mutant phage was digested with *Xba*I and *Sac*I and the mutant F gene ligated into *Xba*I, *Sac*I cut pSVL. The mutations isolated are shown in Fig. 1. Deletion mutants are denoted by the letter d as well as the amino acid positions deleted. For example mutant d523–553 is missing amino acids 523 to 553. Point mutants are named, in single letter code, with the amino acid in wild type, the position of the change, and the amino acid in the mutant. Thus C514S is a mutant which has a cysteine residue substituted with a serine residue at amino acid 514 in the F protein sequence.

Transfections

Transfections using Lipofectin were done essentially as recommended by the manufacturer, BRL/Gibco. Cos cells were plated at 3×10^5 per 35-mm plate. Twenty to 24 hr later, the cells were transfected. For each 35-mm plate, a mix of 2 μg DNA in 0.1 ml OptiMem (BRL/Gibco) and 10 μg of Lipofectin in 0.2 ml of OptiMem was incubated at room temperature for 15 min and then diluted with 0.7 ml OptiMem and added to a plate previously washed with OptiMem. Cells were incubated with the Lipofectin-DNA for 4 to 5 hr and then 2 ml of Cos cell media was added. Transfection efficiency was from 10 to 15%.

Transfections using DEAE dextran/chloroquine were done essentially as previously described (Levesque *et al.,* 1991). Cos cells plated as described above were incubated with a mix of 0.5 ml Tris-buffered saline (0.05 *M* Tris, pH 7.5, 0.15 *M* NaCl) containing 0.1% dextrose, 2.5 μl DEAE/dextran (100 mg/ml in 0.15 *M* Tris–HCl, pH 7.5) and 1 μg DNA for 30 min at 37°. The DNA/dextran mix was replaced with 100 μm chloroquine in 1 ml Opti-Mem (BRL/Gibco) or in Cos media and the cells were incubated for 4 to 5 hr at 37°. The chloroquine was replaced with 2 ml of Cos media. Transfection efficiency was from 40 to 60%.

Antibodies

Antibodies used for immunofluorescent detection and immunoprecipitation of the fusion protein were anti-Fu1a, anti-Fu2a, and anti-NDV antibody. Anti-Fu1a and anti-Fu2a are conformation sensitive monoclonal antibodies previously described (Morrison *et al.,* 1987) and obtained from Dr. Mark Peeples. Anti-NDV is a polyclonal antisera raised in rabbits against uv-inactivated virions as previously described (Sergel *et al.,* 1993). This sera recognizes only mature fusion protein (Sergel *et al.,* 1993).

Immunofluorescence

Cos cells were plated on 35-mm plates containing glass cover slips (Corning) and transfected as described above. The cells were washed twice with phosphate-buffered saline (PBS) and incubated at 4° in PBS containing 3% BSA, 0.02% sodium azide, and anti-NDV antibody (diluted 1:100) for 1 hr. Cells were washed three times with PBS containing BSA and azide and incubated with PBS containing BSA, azide, and fluorescein-conjugated anti-rabbit IgG (Southern Biotechnology) for 1 hr. Cells were washed and a drop of DABCO (25 mg/ml in PBS) was added.

Radiolabeling and immunoprecipitation of protein

Transfected cells were radiolabeled for 2 to 4 hr at 37° in Dulbecco's modified Eagle's medium lacking methionine but containing 100 μCi of [^{35}S]methionine (Amersham) per milliliter and then chased in nonradioactive media for 12 hr. At the end of the labeling period, cells were washed in PBS and lysed in RSB buffer (0.01 *M* Tris–HCl, pH 7.4, 0.01 *M* NaCl) containing 1% Triton X-100 and 0.5% sodium deoxycholate and 2 mg/ml iodoacetamide as previously described (Sergel *et al.,* 1993; Sergel-Germano *et al.,* 1994). Nuclei were removed by centrifugation. Immunoprecipitation of NDV proteins was accomplished as previously described (Sergel *et al.,* 1993; Sergel-Germano *et al.,* 1994).

Syncytia formation

Cos cells were cotransfected with wild-type or mutant fusion protein genes and the wild-type HN protein gene using Lipofectin. At 24, 48, and 72 hr post-transfection, the number of nuclei in 40 fusion areas were counted to determine the average size of syncytia at each time point as previously described (Sergel *et al.,* 1993; Sergel-Germano *et al.,* 1994). Values obtained after transfection of the vector alone were subtracted. All experiments included a control with wild-type HN and F DNAs as well as vector alone. As previously described, this measure of fusion activity is independent of transfection efficiency (Sergel *et al.,* 1993).

Detection of cell surface molecules with antibody

Cells transfected for 48 hr using DEAE dextran protocol were radioactively labeled with [^{35}S]methionine for 2 hr and then chased with nonradioactive media for 16 hr. Anti-NDV antibody was incubated with intact cells as previously described (Morrison *et al.,* 1990). Immune complexes were electophoresed on 10% polyacrylamide gels. The resulting autoradiographs were scanned with a microdensitometer to quantitate the amount of F protein precipitated. Mixing experiments accomplished as previously described guaranteed that all labeled fusion protein precipitated was cell surface fusion protein. All experiments included a wild-type control.

RESULTS

Truncations of the cytoplasmic domain

To determine if the cytoplasmic domain influences the activity of the fusion protein, four mutants missing various amounts of the cytoplasmic domain were constructed by introducing polypeptide chain termination mutations. Diagrammed in Fig. 1, one mutant, d523–553, deleted 31 amino acids or the entire cytoplasmic domain. Mutant d540–553 deleted the fourteen most carboxy terminal amino acids, while mutant d547–553 deleted seven residues and mutant d550–553 deleted the last four residues.

Mutant DNAs were expressed in Cos cells using an SV40 vector and the resulting proteins precipitated with a conformation sensitive monoclonal antibody specific for the NDV fusion protein (anti-Fu1a). Figure 2 shows the resulting precipitated protein electrophoresed in the absence (A) or presence of reducing agent (B). All deletion mutant proteins were precipitated by the antibody, and the migration of the nonreduced forms of the protein were consistent with the deletion of various amounts of the cytoplasmic domain. Electrophoresis in the presence of reducing agent disrupts the linkage between the F_1 and F_2 polypeptides and F_1 and F_0 are resolved on the gel while F_2 is not detected under these conditions (Morrison *et al.,* 1985). Mutant d523–553, missing the entire cytoplasmic domain, shows minimal proteolytic cleavage

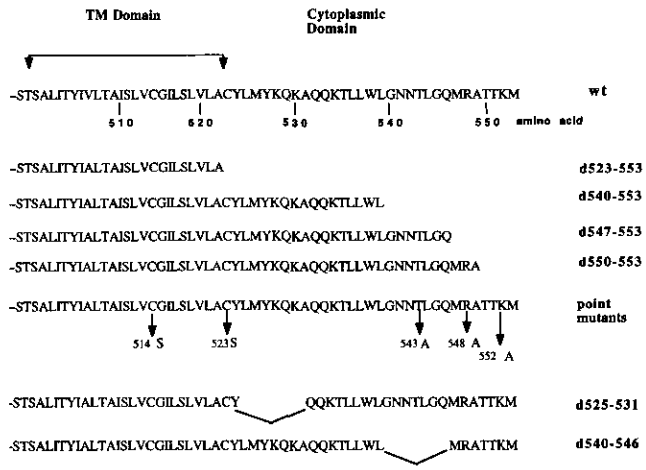

FIG. 1. Location of mutants. The sequence of the transmembrane and cytoplasmic domains of the wild-type fusion protein of NDV (strain AV) is shown in the top line. The sequences of the truncation mutants are shown with the amino acids deleted indicated at the right. The position of the point mutations and the amino acids substituted are indicated by the arrows. Internally deleted sequences are indicated by gaps.

(B). Predominantly one species, consistent with a truncated version of F_0, is resolved. However, d540–553, d547–553, and d550–553 mutant DNAs (B) resulted in proteins that were cleaved at least as efficiently as wild-type protein. Identical results were obtained with anti-NDV antisera (not shown).

To determine if the mutant proteins were expressed at the cell surface, intact cells transfected with these mutants were characterized by immunofluorescence (Figs. 3 C–F). Clearly all truncation mutants could be detected at the cell surface. Furthermore, the percentage of positive cells detected after transfection with each mutant was similar to that detected after transfection of wild-type DNA. Similar results were obtained using two different monoclonal antibodies specific to the NDV F protein (anti-Fu1a and anti-Fu2a) as well as two different polyclonal anti-NDV antibodies (not shown).

Surface immunoprecipitation using anti-NDV antisera was done to quantitate these results. Cells were subjected to a radioactive label followed by a 12-hr chase to minimize differences due to different kinetics of intracellular transport. Figure 4 shows that all truncation mutants except d523–553 were detected at levels comparable to the wild-type protein. Below each lane is the amount of protein detected relative to the wild type. Rather surprising was the absence of significant precipitation of the d523–553, given the positive results of immunofluorescence. It is possible that surface-associated protein can bind antibody, but upon cell lysis the epitopes binding the antibody are disrupted decreasing the precipitation by the antibody.

We and others have previously reported that a population of F protein is often seen as an SDS-resistant oligomer, particularly under nonreducing conditions (Sergel-

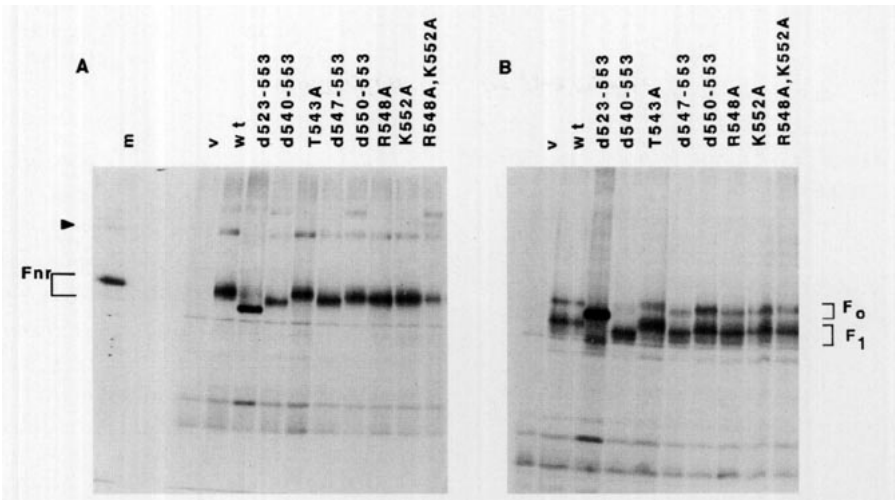

FIG. 2. Immunoprecipitation of truncation and point mutants. Cos cells transfected with vector (v), wild-type F DNA (wt), and mutants were radioactively labeled with [^{35}S]methionine for 2 hr at approximately 48 hr post-transfection and then subjected to a nonradioactive chase for 12 hr. Cell extracts (from 5 × 10^5 cells) were precipitated with anti-Fu1a as described under Materials and Methods and the resulting precipitated proteins resolved on 10% polyacrylamide gels in the absence (A) or presence (B) of reducing agent. The mutant DNA used for each transfection is indicated at the top of the figure. Fnr, nonreduced fusion protein containing both F_0 and F_1 + F_2; F_0, uncleaved fusion protein; F_1, the cleaved fusion protein. F_2 is not resolved on this gel. Arrowhead indicates the SDS resistant oligomer (see text).

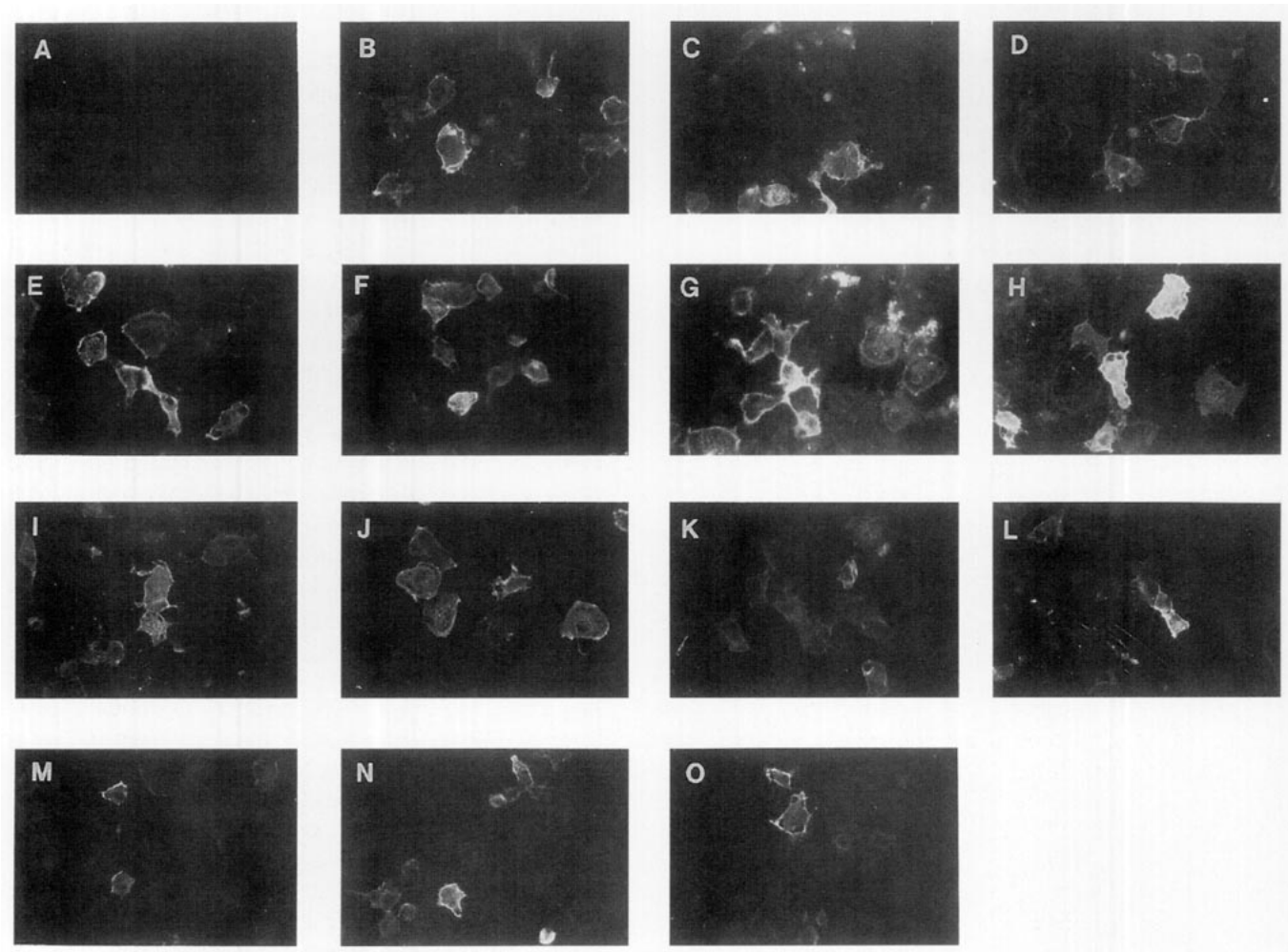

FIG. 3. Immunofluorescent detection of surface expression of mutant proteins. At 48 hr post-transfection, intact cells transfected with vector DNA (A), wild-type DNA (B), and mutant DNAs were prepared for immunofluorescent detection as described under Materials and Methods. All cells were photographed using the same time of exposure. C, d523–553; D, d540–553; E, d547–553; F, d550–553; G, T543A; H, R548A; I, K552A; J, R548A, K552A; K, d525–531; L, d540–546; M, C514S; N, C523S; O, C514S, 523S.

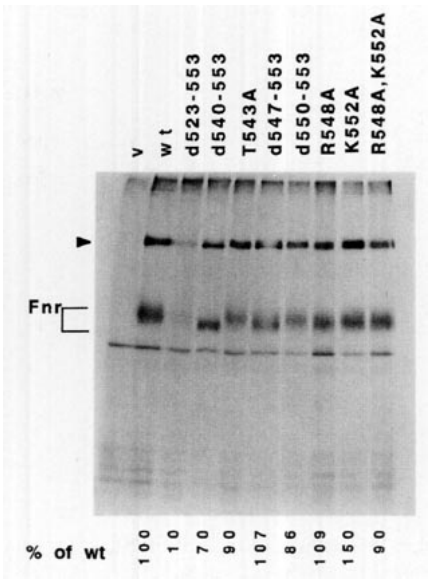

FIG. 4. Immunoprecipitation of cell surface fusion proteins. At 48 hr post-transfection, Cos cells transfected with vector (v), wild-type (wt), or mutant DNAs were radioactively labeled with [^{35}S]methionine for 2 hr and then chased for 12 hr in nonradioactive media. Intact cells were incubated with antibody as described under Materials and Methods. After washing away unbound antibody, cells were lysed and immune complexes precipitated and resolved on 10% polyacrylamide gels in the absence of reducing agent. The mutant DNA used for each transfection is indicated at the top of the panel. At the bottom of the panel, numbers indicate the percentage of protein detected at the surface relative to wild-type protein (100%). The values are the average of three separate experiments. Variation between experiments was at most 20%. Arrowhead indicates the position of the SDS resistant oligomer. Fnr, fusion protein nonreduced.

Germano *et al.*, 1994; Taylor *et al.*, 1993). All mutants formed this oligomeric structure (see arrow, Figs. 3 and 4), except d523–553 which formed much less of this material.

To test the syncytia forming activity of the mutant proteins, cells were transfected with the wild-type or mutant protein DNAs as well as the wild-type HN protein gene which is required to promote the F protein fusion (Morrison *et al.*, 1991; Sergel *et al.*, 1993). Syncytia forming activity of each fusion protein mutant was measured as the average size of syncytia formed with time after transfection (Fig. 5A). Mutant d523–553 was devoid of activity, a result consistent with inefficient cleavage of the mutant protein. Mutant d540–553, which is proteolytically cleaved, was also defective in activity. Mutant d547–553 had activity although reduced from wild type, while mutant d550–553 had wild-type levels of activity.

These results suggest two possible explanations. First, a sequence located in between amino acid 540 and 550 may be important to the activity of the protein. Alternatively, a length of 24 to 26 amino acids is required and not a specific sequence. To test these two possibilities, point mutations were introduced into the cytoplasmic domain in between amino acids 542 and 552. In addition, two internal deletions of 7 amino acids were introduced into the cytoplasmic domain sequence to result in a cytoplasmic domain of 24 amino acids.

Point mutations

Three point mutations were introduced into the carboxy terminal region of the cytoplasmic domain (Fig. 1). Threonine at position 543, arginine at position 548, and lysine at position 552 were all changed individually to an alanine to produce T543A, R548A, and K552A, respectively. In addition, a double mutant R548A, and K552A was also constructed. The proteins expressed from these mutant DNAs were characterized by precipitation with anti-Fu1a monoclonal antibody (Fig. 2). Clearly, all four proteins were precipitated by the antibody and all proteins were proteolytically cleaved at least as efficiently as the wild-type protein. In addition, all proteins were detected at the cell surface by immunofluorescence (Figs. 3G–J) and by cell surface immunoprecipitation (Fig. 4) at levels comparable to wild type.

The syncytia forming activity of these point mutants is shown in Fig. 5B. While K552A, R548A, and the double mutant containing both changes have wild-type or nearly wild-type levels of fusion activity, the mutant T543A has considerably less activity than wild-type protein. This result suggests that the length of the cytoplasmic domain is not the only requirement for syncytia formation but that the sequence, likely between amino acid 540 and 548, is of some importance.

Internal deletions

This possibility was further explored by constructing two internal deletions (Fig. 1), one of which deleted seven amino acids from the more amino terminal region of the cytoplasmic domain (d525–531), while the other deleted a more carboxy terminal region (d540–546). Proteins expressed from these mutant DNAs were immunoprecipitated with anti-NDV antisera, a conformation-sensitive antibody (Sergel *et al.*, 1993). The precipitated proteins were electrophoresed in the absence and presence of reducing agent (Figs. 6A and 6B). Both mutant proteins were precipitated and both were proteolytically cleaved. In addition, both mutant proteins were expressed at the cell surface at levels comparable to the wild type as determined by immunofluorescence (Figs. 3K and 3L) and surface precipitation (Fig. 6C).

The syncytia forming activity of these mutant proteins is shown in Fig. 7. Mutant d525–531 has only slightly decreased activity. However, the activity of mutant d540–546 is considerably reduced, to 25 to 30% of wild type, again suggesting the importance of the sequence from amino acid 540–550.

Mutation of potential palmitate addition sites

Some glycoproteins, including the NDV fusion protein, are modified by fatty acid palmitate (Chatis and Morrison, 1982). In several systems, it has been shown that palmi-

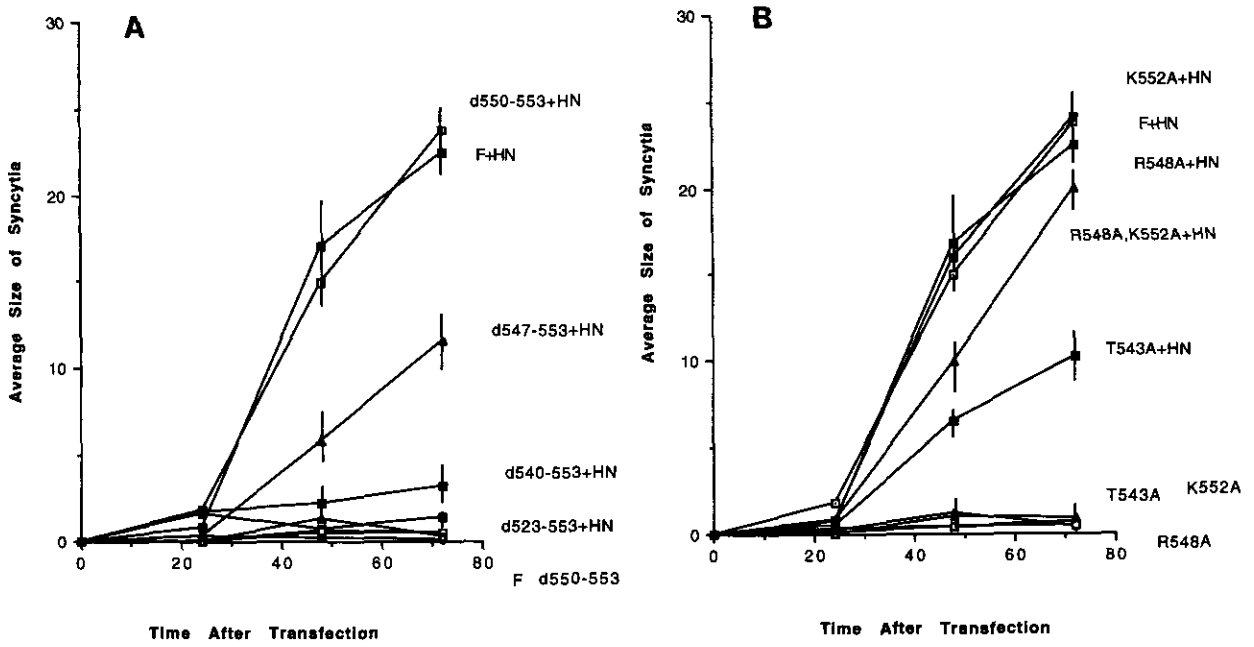

FIG. 5. Fusion activities of truncation and point mutants. Cos cells were cotransfected with wild-type HN protein DNA and wild-type or mutant F protein DNAs. At 24, 48, and 72 hr post-transfection, fusion was assayed as described under Materials and Methods. All assays were performed at least three times and the average values obtained are shown. Also shown by the vertical lines at each point is the actual variation observed in all experiments with each mutant at each time point. A shows results with truncation mutants and B shows results with point mutants.

tate is covalently added to cysteine residues in and near the transmembrane domain (Koch and Hammerlin, 1986; Rose *et al.*, 1984; Schmidt *et al.*, 1988; Veit *et al.*, 1991a). To determine if palmitate addition might play any role in syncytia formation, the cysteine residues at amino acid 514 and 523 were changed to serine individually to produce mutants C514S and C523S and in combination to produce C514S,523S (Fig. 1). All three mutant proteins were precipitated by anti-NDV antisera (Fig. 8A) and all three proteins were proteolytically cleaved (Fig. 8B). In-

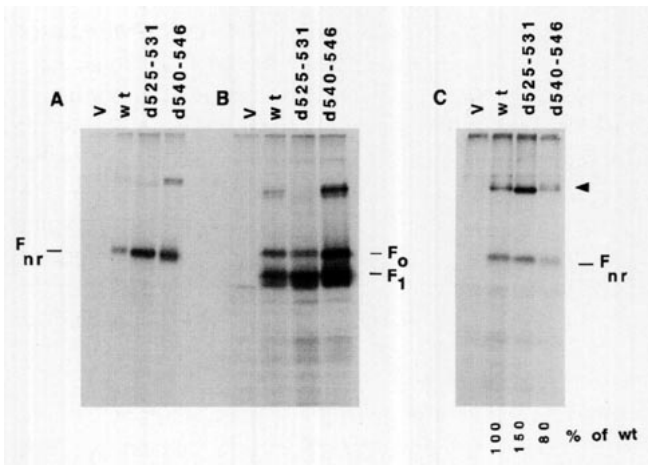

FIG. 6. Immunoprecipitation of proteins with internal deletions. Cos cells transfected with vector (v), wild-type F (wt) DNA, d525–531, or d540–546 were radioactively labeled as described in the legend to Fig. 2. A and B show immunoprecipitation of total cell extracts with anti-NDV antisera electrophoresed in the absence (A) and presence (B) of reducing agent. C shows precipitates of cell surface molecules prepared as described in the legend to Fig. 4. The numbers under the lanes in C are the average values relative to wild type obtained in three separate experiments. Fnr, nonreduced fusion protein containing both F_0 and $F_1 + F_2$; F_0, uncleaved fusion protein; F_1, the cleaved fusion protein.

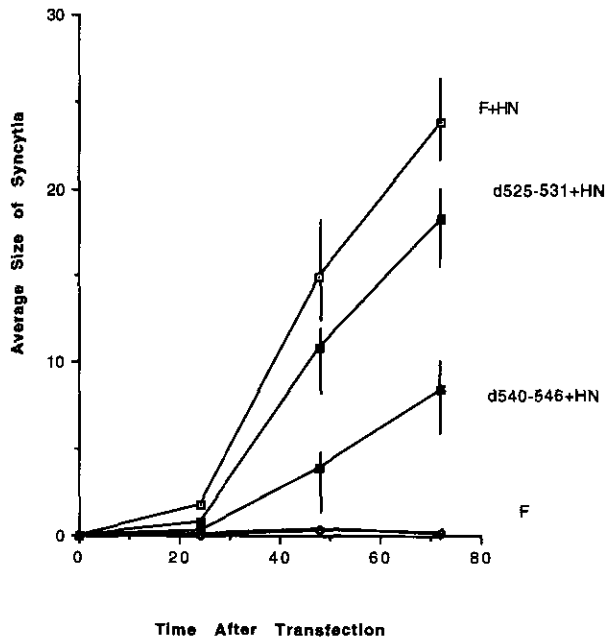

FIG. 7. Fusion activities of proteins with internal deletions. Cos cells were cotransfected with wild-type HN protein DNA and wild-type F protein DNA or mutant DNAs and the average size of syncytia at 24, 48, and 72 hr post-transfection was determined as described under Materials and Methods. The results are the averages of four separate experiments.

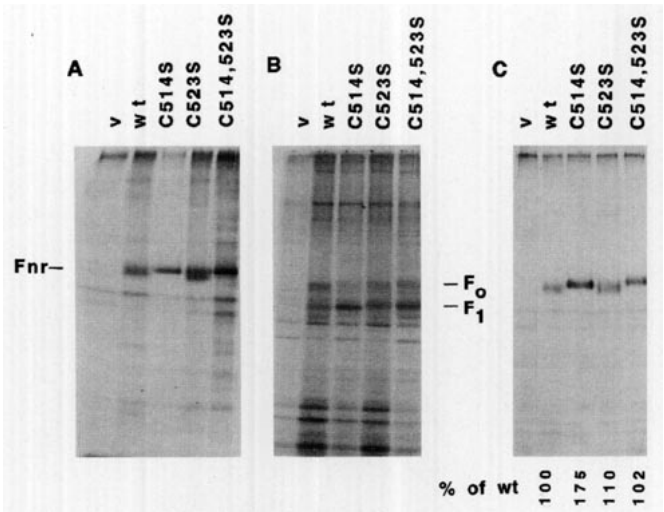

FIG. 8. Immunoprecipitation of cysteine mutants. Cos cells transfected with vector (v), wild-type (wt), C514S, C523S, and C514S,523S DNA were radioactively labeled with [^{35}S]methionine as described in the legend to Fig. 2. Protein in total cytoplasmic extracts was precipitated with anti-NDV antisera and the precipitates electrophoresed in the absence (A) or presence (B) of reducing agent. Cell surface molecules (C) bound to antibody were precipitated as described in the legend to Fig. 4. The numbers under the lanes in C represent the average values obtained relative to wild type for each mutant in three separate experiments. Fnr, F nonreduced; F_0, uncleaved fusion protein; F_1, cleaved fusion protein.

terestingly, the proteins containing the C514S mutation migrated on gels slightly slower than the wild-type protein and as a more discrete band then is typical of the wild-type protein.

All three proteins were also expressed at the cell surface at wild-type levels (Figs. 3M–O and Fig. 8C). In addition, all three mutant proteins showed nearly wild-type levels of syncytia forming activity (Fig. 9). Thus there is no evidence that these residues influence the activity of the protein.

DISCUSSION

Cytoplasmic domain and conformation of ectodomain

Mutation d523–553 eliminates the entire cytoplasmic domain of the fusion protein. The protein expressed by this mutant DNA is recognized by a conformation-sensitive monoclonal antibody as well as polyclonal antibody that is conformation sensitive; thus, the mutant protein has some mature conformational determinants. However, the protein is not proteolytically cleaved, a modification of the protein which occurs in the trans Golgi membranes (Morrison *et al.*, 1985). Failure to cleave in the absence of alterations in the ectodomain of the protein may indicate either an altered conformation masking the cleavage site or inefficient transport to the site of cleavage also likely due to an inappropriate folding. The mutant protein could be detected at the cell surface by immunofluorescence at a frequency and intensity similar to wild-type protein using three different antibodies. However, rather unexpectedly, the mutant protein was minimally detected by surface immunoprecipitation. A possible explanation is that epitopes recognized by the antibody on intact cells are disrupted upon cell lysis. These results are very different from those obtained with the wild-type protein, suggesting conformational differences from the wild-type protein. Two other results suggest altered conformation. This mutant protein exhibits less SDS-resistant oligomeric material on polyacrylamide gels than wild type. Furthermore, addition of trypsin to extracts containing the mutant protein does not result in cleavage of the molecule under conditions in which an uncleaved, full-length F protein from an avirulent strain of NDV is cleaved (unpublished observations). Thus, in contrast to the full-length protein, the cleavage site of the truncated molecule is inaccessible to added protease. Cells expressing this protein have no syncytia forming activity, a result that may be attributed to defects in the cleavage of the protein as well as conformational alterations.

Cytoplasmic domain and syncytia formation

The mutation d540–553, which deletes 14 amino acids or approximately half of the cytoplasmic domain, demonstrates that the cytoplasmic domain of the fusion protein influences the biological activity of the protein. The protein expressed from this mutant DNA is precipitable by a conformation-sensitive antibody (Morrison *et al.*, 1987) and proteolytically cleaved at least as efficiently as the wild-type protein. Furthermore, the protein is detected at

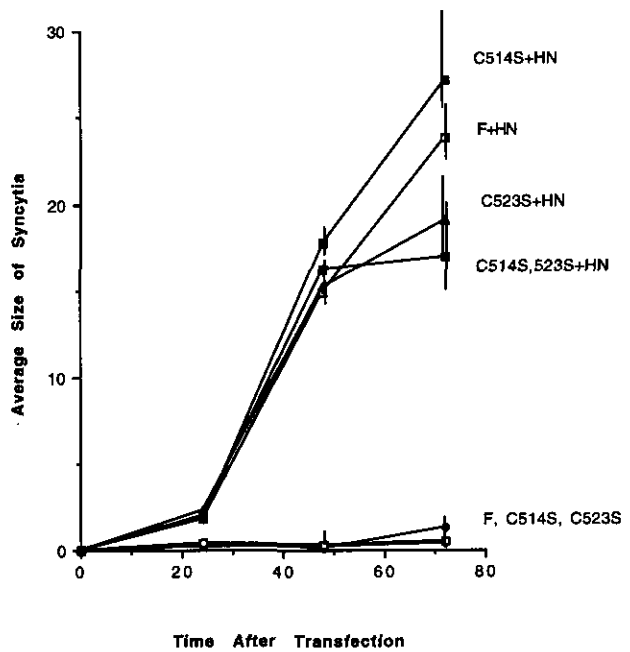

FIG. 9. Fusion activities of cysteine mutants. Cos cells were cotransfected with wild-type HN protein DNA and wild-type F protein DNA or mutant DNAs and the average size of syncytia at 24, 48, and 72 hr post-transfection was determined as described under Materials and Methods. Shown are the averages of three separate experiments.

the cell surface at levels comparable to wild type by both immunofluorescence and surface immunoprecipitation. Thus by these criteria, the mutant protein appears to be conformationally normal. Furthermore, long chase periods used for cell surface immunoprecipitation yielded similar amounts of protein as wild type, suggesting that the mutant protein is as stable as wild type. However, this protein has virtually no syncytia forming activity.

By similar criteria, the other truncation mutants are also conformationally normal. Protein missing seven amino acids from the carboxy terminus has approximately 30% wild-type syncytia forming activity while protein missing four amino acids has wild-type levels of activity. These results suggest that the sequences between amino acids 540 and 550 are particularly important to this activity of the protein. This conclusion is supported by the point mutations in this region as well as the two internal deletions. Deletions of seven amino acids in the more amino terminal region of the domain had little effect on syncytia formation while a similar-sized deletion in the more carboxy terminal region depressed syncytia formation by 70 to 75%.

Comparisons of the primary sequences of paramyxovirus fusion proteins has shown that there are important structural determinants conserved across the entire family (Morrison and Portner, 1991). The position of hydrophobic domains, cysteine residues, glycine residues, and proline residues as well as structural motifs such as heptad repeat regions are remarkably conserved. Furthermore, the primary sequences of the fusion peptides are very conserved. However, comparisons of the cytoplasmic domains show no apparent conservation in primary sequence, distribution of charges, distribution of similar types of amino acids, or even length. No sequence elements found between amino acids 540 and 550 in the NDV F protein sequence are common to other paramyxovirus fusion proteins. Perhaps the cytoplasmic domain interacts with cellular proteins such as cytoskeletal components which participate in syncytia formation. Indeed, it has been shown that truncation of the cytoplasmic domain of the PIV F protein results in a failure of the protein to cap in the presence of antibody (Lydy and Compans, 1993). However, the absence of a common motif in this domain makes this possibility less likely. Alternatively, the cytoplasmic domain may play an important role in the conformation of the fusion protein or in conformational changes that must take place during the fusion process. The domain may also influence the oligomeric structure of the protein required for activity. Oligomer formation is not likely blocked in these mutants since a deletion which removes both the entire cytoplasmic domain and the transmembrane domain results in a protein which still forms oligomeric structures which, like the wild-type protein, sediment on sucrose gradients as a trimer (Reitter, Sergel, and Morrison, submitted). The cytoplasmic domain may, however, affect the conformation of the oligomeric structure or changes in the structure upon activation of the fusion protein by the HN protein.

Measles virus F protein genes derived from brains of different SSPE patients have various alterations in the cytoplasmic domain of the protein (Schmid *et al.,* 1992). Characterization of the syncytia forming ability of several truncated F proteins (Cattaneo and Rose, 1993) has shown that one, with a deletion of the 24 carboxy terminal amino acids (d529–553), has minimal syncytia forming activity. However, another, missing 19 amino acids from the carboxy terminus (d534–553), is active in syncytia formation when combined with a wild-type HA protein. These results are similar to those reported here in that mutants retaining longer portions of the cytoplasmic domain are more active in syncytia formation than mutants with less of the cytoplasmic domain. Thus these two deletions may define a region of the measles virus F protein cytoplasmic domain important for syncytia formation, from amino acids 529 to 534.

Palmitate addition

One modification frequently characteristic of viral glycoproteins is covalent addition of palmitate to cysteine residues near the transmembrane domain (McIlhinney, 1990; Rose *et al.,* 1984; Schmidt *et al.,* 1988; Veit *et al.,* 1991a,b). Indeed, the fusion protein of NDV grown in chick embryo fibroblasts can be labeled with palmitate (Chatis and Morrison, 1982). There has been one report that mutation of these cysteine residues of influenza HA results in impairment of the fusion activity of the protein (Naeve and Williams, 1990). However, similar studies of the HA protein of other strains of influenza showed no effect of this modification on the activity of the protein (Naim *et al.,* 1992; Simpson and Lamb, 1992; Steinhauer *et al.,* 1991; Veit *et al.,* 1991a). The NDV fusion protein has two cysteine residues which are potential palmitate addition sites (Morrison and Portner, 1991), one located within the transmembrane domain and the other at the likely interface between the transmembrane domain and the cytoplasmic domain. Mutation of these two cysteine residues, either singly or in combination, to serine residues had no effect on levels of syncytia formation, thus there is no indication that palmitate addition plays a role in this activity of the NDV F protein. Indeed, attempts to demonstrate fatty acid acylation of the wild-type protein either after transfection of the F protein gene or after infection with NDV in Cos cells was unsuccessful (unpublished observations), raising the possibility that this modification does not occur in all cell types.

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